

# Brefeldin A inhibits protein synthesis through the phosphorylation of the $\alpha$ -subunit of eukaryotic initiation factor-2

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## Abstract

Brefeldin A is a fungal metabolite which disrupts protein traffic through the Golgi apparatus and thereby inhibits protein secretion. Recently, it has been shown that Brefeldin A also causes a marked decrease in the rate of protein synthesis in cells in culture [1992, FEBS Lett. 314, 371–374]. We show here that treatment of rat GH3 pituitary cells with Brefeldin A leads to an inhibition of protein synthesis at the level of peptide-chain initiation through a mechanism involving the phosphorylation of the  $\alpha$ -subunit of eukaryotic initiation factor-2 (eIF-2 $\alpha$ ).

**Key words:** Brefeldin A; Protein synthesis; eIF-2; Protein phosphorylation

## 1. Introduction

Brefeldin A is a fungal metabolite which causes the collapse of the Golgi apparatus into the endoplasmic reticulum (ER) resulting in a total inhibition of protein secretion [1]. Disassembly of the Golgi apparatus is accompanied by a redistribution of resident Golgi membrane proteins to the ER [2] and an accumulation of incompletely-processed glycoproteins in this compartment ([3,4], reviewed in [5]). It has recently been shown that Brefeldin A has an inhibitory effect on protein synthesis in a wide variety of cells in culture over the same concentration range used to inhibit protein secretion [6]. The nature of this inhibition has been unclear. It has previously been reported that certain inhibitors of ER-processing cause an inhibition of protein synthesis through a mechanism involving the phosphorylation of the  $\alpha$ -subunit of eukaryotic initiation factor-2 (eIF-2 $\alpha$ , [7] and refs. therein). eIF-2 mediates the binding of the initiator tRNA (Met-tRNA<sub>i</sub>) to the 40S ribosomal subunit in eukaryotic cells (reviewed in [8]). This reaction is dependent on GTP which is hydrolyzed to GDP prior to the association of the 60S ribosomal subunit. eIF-2 is then released from the ribosome as an inactive complex with GDP. The guanine nucleotide-exchange factor eIF-2B catalyzes the exchange of GDP for GTP on eIF-2, thus regenerating active eIF-2. Phosphorylation of eIF-2 $\alpha$  at a specific serine residue (Ser<sup>51</sup>) impairs this exchange reaction and leads to an inhibition of translational initiation [8]. We show here that Brefeldin A-treatment of rat GH3 pituitary tumor cells leads to an inhibition of protein synthesis at the level of peptide-chain initiation in parallel with an increase in eIF-2 $\alpha$  phosphorylation and a decrease in eIF-2B activity.

## 2. Materials and methods

### 2.1. Materials

Brefeldin A was obtained from Sigma and was stored as a 5 mg·ml<sup>-1</sup> solution in ethanol at -20°C. [<sup>3</sup>H]GDP and cell-labeling grade [<sup>35</sup>S]methionine were from Amersham. Ampholytes (BDH-certified Re-solute, pH 4–8) were from Hoefer Scientific Instruments.

### 2.2. Cell culture

Rat GH3 pituitary tumor cells were grown in culture in Dulbecco's Modified Eagle Medium containing 25 mM HEPES (Life Sciences Inc.) supplemented with 10% fetal bovine serum (Hyclone Labs. Inc.), 100 U·ml<sup>-1</sup> benzylpenicillin and 100  $\mu$ g·ml<sup>-1</sup> streptomycin sulfate.

### 2.3. Ribosomal subunit analysis

Cells (approximately  $5 \times 10^6$  cells) were washed in ice-cold wash buffer (5 mM Tris-HCl, pH 7.4, 1.5 mM KCl, 2.5 mM MgCl<sub>2</sub>) and extracted by a modification of the method of Thomas et al. [9]. The washed cells were lysed in 1 ml of ice-cold extraction buffer (50 mM Tris-HCl, pH 7.4, 26 mM KCl, 5 mM MgCl<sub>2</sub>, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 4  $\mu$ g·ml<sup>-1</sup> leupeptin and 4  $\mu$ g·ml<sup>-1</sup> pepstatin). The extracts were then clarified by centrifugation at  $10,000 \times g$  for 10 min at 4°C and 500  $\mu$ l of each extract was layered onto linear 20–47% (w/v) sucrose gradients. The gradients were centrifuged at  $200,000 \times g$  for 2 h at 4°C. The gradients were then analyzed by upward displacement through the optical cell of an ISCO 640 density gradient fractionator which recorded the absorbance at 254 nm.

### 2.4. Measurement of protein synthesis and eIF-2 $\alpha$ phosphorylation

Cells (approximately  $0.5 \times 10^6$  cells) were incubated in culture medium in the presence or absence of 1  $\mu$ g·ml<sup>-1</sup> Brefeldin A. For the measurement of protein synthesis, cells were incubated in the presence of [<sup>35</sup>S]methionine (approximately 5  $\mu$ Ci·ml<sup>-1</sup>). At various time points, the culture medium was removed and the cells were washed once with 1 ml of ice-cold phosphate-buffered saline and then twice with 1 ml of ice-cold 10% (w/v) trichloroacetic acid. The acid-washed cell pellets were then dissolved in 0.2 M NaOH and the incorporation of radiolabel into protein was determined by liquid scintillation counting. For the measurement of eIF-2 $\alpha$  phosphorylation, cells were extracted in 200  $\mu$ l of SDS-PAGE sample buffer at 90°C. The samples were heated in 90°C for 3 min, diluted with 2 vols. of isoelectric focusing gel buffer (9.5 M urea, 4% (v/v) ampholytes, 5% (v/v) 2-mercaptoethanol, 8% Nonidet P-40) and then adjusted to a final urea concentration of 9.5 M. The proportion of phosphorylated eIF-2 $\alpha$  was determined by protein immunoblot analysis of slab isoelectric focusing gels using a monoclonal antibody to eIF-2 $\alpha$  (generously provided by Drs. R. Panniers and E.C. Henshaw) according to the protocol of Kimball and Jefferson [10].

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### 2.5. Measurement of eIF-2B activity

Cells (approximately  $1 \times 10^6$  cells) were incubated in culture medium in the presence or absence of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A for 3 h. The culture medium was removed and the cells were washed once with ice-cold phosphate-buffered saline and then extracted with  $400 \mu\text{l}$  of ice-cold extraction buffer (45 mM HEPES, pH 7.4, 0.375 mM magnesium acetate, 0.075 mM EDTA, 95 mM potassium acetate,  $2.5 \text{ mg} \cdot \text{ml}^{-1}$  digitonin and 10% (v/v) glycerol). The extracts were clarified by centrifugation at  $10,000 \times g$  for 10 min at  $4^\circ\text{C}$  and the supernatants were then assayed for the exchange of  $[^3\text{H}]\text{GDP}$  bound to eIF-2 for unlabeled GTP as previously described [11].

### 2.6. Quantification of eIF-2 and eIF-2B

The molar ratio of eIF-2:eIF-2B in GH3 cells was determined as described [12] by immunoblot analysis of cell extracts using monoclonal antibodies against rat eIF-2 $\alpha$  and the  $\epsilon$ -subunit of eIF-2B. Experiments were performed in triplicate and the signals were quantitated by comparison with standard samples of purified rat eIF-2 and eIF-2B.

## 3. Results

### 3.1. Inhibition of peptide-chain initiation by Brefeldin A

We examined the effect of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A on the rate of protein synthesis in GH3 cells. This concentration was chosen because it has been widely used to inhibit Golgi function in studies of protein secretion. Incubation of GH3 cells in the presence of Brefeldin A led to an approximately 33% inhibition of protein synthesis (Fig. 1), a level of inhibition similar to that reported with other cell-types [6]. Inhibition of protein

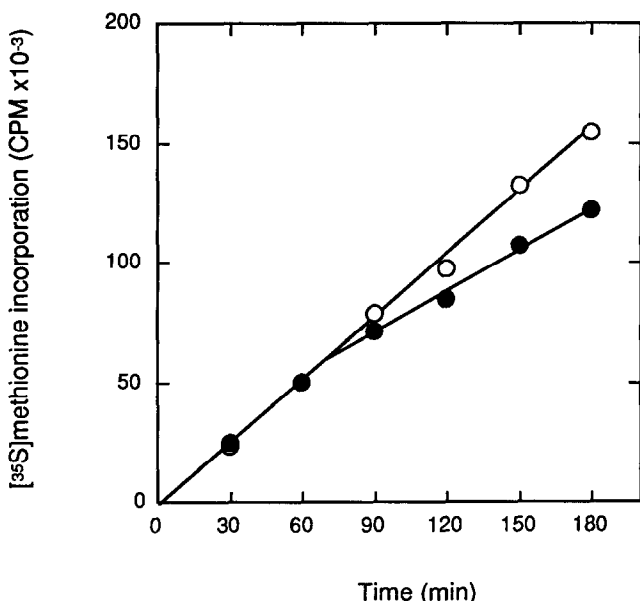


Fig. 1. Cells were incubated with  $[^3\text{S}]\text{methionine}$  in the absence (○) or presence (●) of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A. At the times indicated the incorporation of radiolabel into protein was measured as described in section 2.4. Data represent the mean  $\pm$  S.E.M. of experiments performed in triplicate (the error bars fall within the size of each point). The standard error on each data point was  $< 5\%$ . Cells incubated in the absence of Brefeldin A incorporated  $[^3\text{S}]\text{methionine}$  into protein at a rate of  $856 \text{ cpm} \cdot \text{min}^{-1}$ . This rate of protein synthesis was matched in cells treated with Brefeldin A until approximately 90 min of incubation, when the rate of incorporation of radioactivity decreased to  $573 \text{ cpm} \cdot \text{min}^{-1}$ .

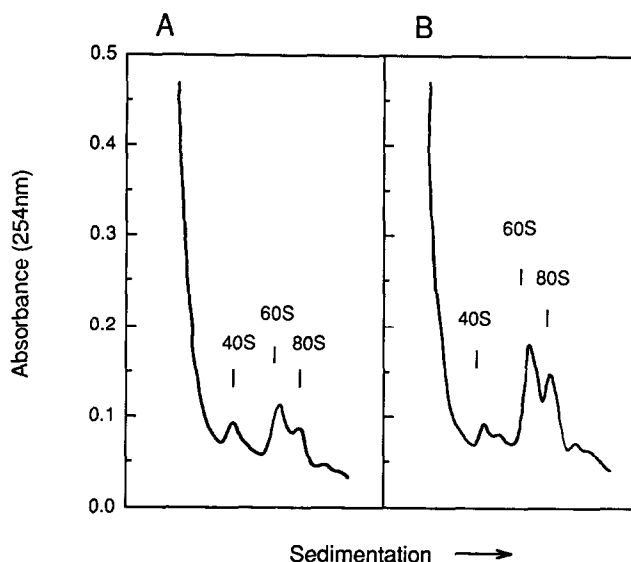


Fig. 2. Cells were incubated in the absence (Panel A) or presence (Panel B) of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A for 3 h. The cells were extracted and the post-mitochondrial supernatants were analyzed by density-gradient centrifugation to examine the state of ribosomal aggregation. The figure shows a representative experiment. The positions of the 40S and 60S ribosomal subunits and the 80S monomer are indicated. Brefeldin A-treatment caused a 48% increase in free 60S ribosomal subunits and 80S monomers (average of duplicate experiments).

synthesis was not observed until 90 min of incubation suggesting that this was a consequence of inhibition of protein secretion, which occurs over a similar time course in these cells [13]. Density gradient analysis of the state of ribosomal aggregation in Brefeldin A-treated GH3 cells showed that the inhibition of protein synthesis occurred at the level of peptide-chain initiation, as indicated by a marked increase in free ribosomal subunits and monomeric ribosomes relative to control cells (Fig. 2).

### 3.2. Brefeldin A-treatment leads to an increase in eIF-2 $\alpha$ phosphorylation

The involvement of eIF-2 $\alpha$  phosphorylation in the control of protein synthesis by other modulators of ER processing prompted us to examine the state of phosphorylation of this initiation factor in Brefeldin A-treated cells. Brefeldin A caused a time-dependent increase in eIF-2 $\alpha$  phosphorylation which paralleled the inhibition of protein synthesis (Fig. 3). Phosphorylation was first observed at 60 min and quickly plateaued at a level where approximately 25% of the factor was in the phosphorylated state (Fig. 3). After 3 h of Brefeldin A treatment, when 26% of eIF-2 $\alpha$  is phosphorylated, the nucleotide-exchange activity of eIF-2B in cell extracts (calculated over the time course of linear response) was found to be reduced by approximately 41% (Fig. 4). This reduction is in reasonable agreement with the effect on protein synthesis (33% inhibition) but out of proportion

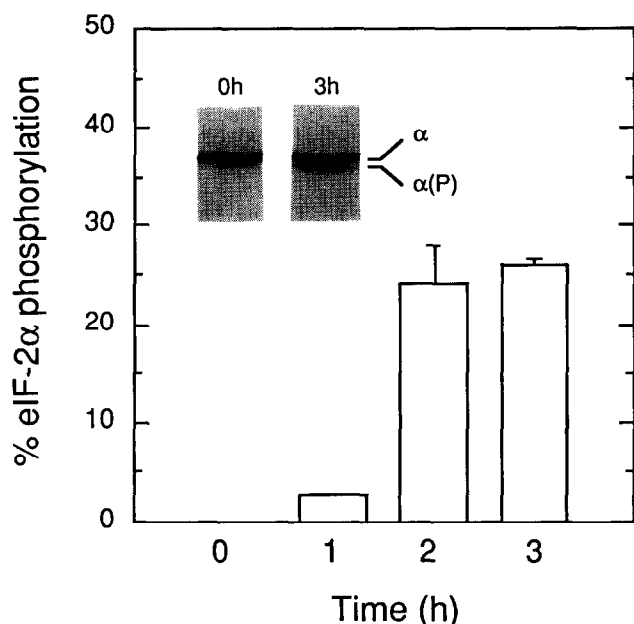


Fig. 3. Cells were incubated in the presence of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A. At the times indicated samples were extracted and the proportion of eIF-2 $\alpha$  in the phosphorylated state was determined by protein immunoblot analysis of isoelectric focusing slab gels as described in section 2.4. Data represent the mean  $\pm$  S.E.M. of experiments performed in triplicate. The inset panels show the immunoblots of the 0 h and 3 h samples from a representative experiment.

with the change in eIF-2 $\alpha$  phosphorylation. Previous studies have used indirect methods to measure the molar ratios of eIF-2:eIF-2B in rabbit reticulocytes (6:1, [14]) and Ehrlich ascites cells (2:1, [15]) and it has been proposed that a consequence of these high molar ratios is that only a small increase in eIF-2 $\alpha$  phosphorylation is required to cause a significant inhibition of eIF-2B activity [8]. In recent studies, we ([12] and unpublished data) and others [16] have directly quantified the amounts of eIF-2 and eIF-2B in various tissues and demonstrated considerable tissue-specific variation. It is therefore important to determine the ratio of eIF-2:eIF-2B in any system when evaluating the relevance of changes in eIF-2 $\alpha$  phosphorylation. We determined the molar ratio of eIF-2:eIF-2B in GH3 cells to be  $1.3:1 \pm 9.4\%$  (S.E.M.,  $n = 3$ , Fig. 5). Brefeldin A-treatment did not effect this ratio, nor the cellular level of either factor (data not shown). Phosphorylation of 26% of the eIF-2 $\alpha$  after 3 h of Brefeldin A-treatment would therefore be expected to lead to a 34% inhibition of eIF-2B activity and a similar degree of inhibition of protein synthesis, in good agreement with our experimental findings.

#### 4. Discussion

In the studies presented here we have shown that the inhibition of protein synthesis in Brefeldin A-treated

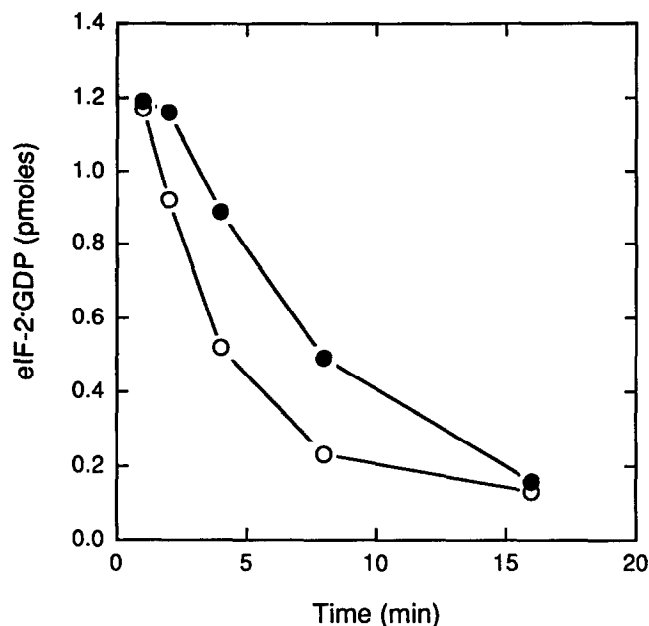


Fig. 4. Cells were incubated in the absence (○) or presence (●) of  $1 \mu\text{g} \cdot \text{ml}^{-1}$  Brefeldin A for 3 h. The cells were then extracted and assayed for eIF-2B activity as described in section 2.5. The figure shows the amount of eIF-2·[ $^3\text{H}$ ]GDP complex remaining over a 16 min time course. Data represent the average of two experiments.

GH3 cells occurs at the level of peptide-chain initiation and is accompanied by an increase in eIF-2 $\alpha$  phosphorylation and a decrease in eIF-2B activity. Our studies were prompted by research which has shown that eIF-2 $\alpha$  phosphorylation increases when the ER is depleted of calcium by calcium-mobilizing hormones such as vasopressin [17] or chemical agents [7,10,18,19], or when the redox state of this compartment is altered by treatment

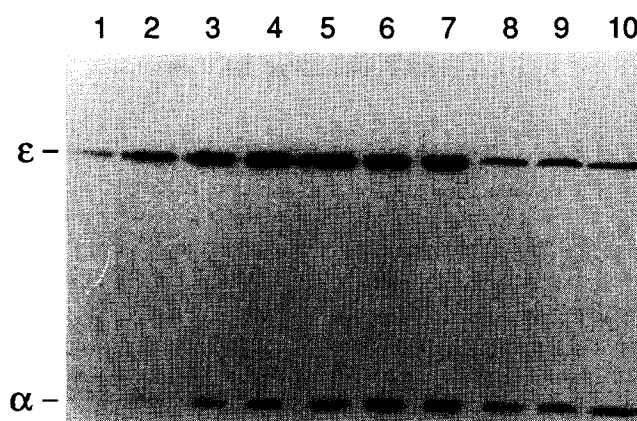


Fig. 5. The molar ratio of eIF-2 to eIF-2B in GH3 cells was determined by immunoblot analysis of cells extracts separated by SDS-PAGE as described in section 2.6. Lanes 1–7; 2.4, 4.8, 7.2, 9.6, 12, 14.4 and 16.8 ng of purified eIF-2, respectively and, 8, 16, 24, 32, 40, 48 and 56 ng of purified eIF-2B, respectively. Lanes 8–10 contain approximately  $14 \mu\text{g}$  of cell extract from three separate GH3 cell cultures. The positions of the  $\alpha$ -subunit of eIF-2 and the  $\epsilon$ -subunit of eIF-2B are indicated.

with dithiothreitol [7]. These conditions lead to an accumulation of partially-processed and misfolded proteins in the ER [7] as does Brefeldin A treatment [3,4]. Inhibitors of glycoprotein processing which do not cause the retention of protein by the ER do not trigger eIF-2 $\alpha$  phosphorylation or an inhibition of protein synthesis [7]. eIF-2 is a cytosolic protein and clearly a mechanism must exist to sense an accumulation of protein in the ER and communicate this to the cytoplasmic compartment. Prostko et al. [7] have suggested that the signal may be generated at the site of the paused translocon on the exterior face of the ER, however, the nature of this signal is obscure. Heme-controlled repressor (HCR) is a serine/threonine protein kinase whose only known substrate is eIF-2 $\alpha$  [20]. It has been suggested that this protein kinase may be responsible for the increase in eIF-2 $\alpha$  phosphorylation observed during heat shock and, in support of this hypothesis, it has been shown that HCR is activated when denatured protein is added to rabbit reticulocyte lysates [21]. HCR may be similarly activated by nascent peptide chains frozen at the translocation site on the ER by inhibitors of ER-processing. In this case, inhibition of protein synthesis would initially be localized to this area. This would explain the loss of ribosomes from the ER surface seen in Brefeldin A-treated cells [22]. We are currently investigating the possible involvement of HCR and other eIF-2 $\alpha$  kinases in the inhibition of peptide-chain initiation caused by Brefeldin A-treatment.

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